CHAPTER 3:

NF-κB, Hela-Tat and cytotoxicity assays on plant extracts

3.1 Introduction

The human immunodeficiency virus type 1 (HIV-1) infects CD4+ T-lymphocytes and macrophages and its genetic material is integrated into the infected cell's genome. Upon integration the virus remains transcriptionally silent and this allows the infected cell to escape currently used antiretroviral drugs. In latently infected cells, viral transcription can be reactivated with various stimuli, including, phorbol esters and cytokines (Marcello *et al*., 2004). When cells are activated, transition from latency to HIV gene expression occurs and requires the converted action of cellular transcription factors and regulatory HIV proteins (Bedoya *et al*., 2005). Among these proteins the HIV cellular transcription factor NF-κB and Tat are required for efficient HIV-1 replication. These proteins regulate the post-integration phase of the viral cycle, which preferentially occurs in activated cells on the long terminal repeat promotor (LTR). The viral regulatory proteins and cellular factors represent potential targets that should be considered in the search for anti-HIV-1

agents, because they determine the extent of HIV-1 gene transcription and the level of viral replication in the infected cells (Sancho *et al*., 2004).

Because of a persistent and urgent need for a preventive HIV vaccine, interest in the anti-HIV activity of traditional medicinal plants has now gained momentum. Because of their low cost, plants have been increasingly explored for production of biomedicine and vaccines (Karasev *et al*., 2005). Several plant-derived substances including phenylcoumarins and plant proteins have showed good anti-HIV activity that can be related to inhibition of NF-κB and Tat proteins (Akesson *et al*., 2003; Reddy *et al*., 2004; Marquez *et al*., 2005). This part of the study is aimed at evaluating anti-NF-κB and anti-Tat activity and cytotoxicity of medicinal plant extracts that are used traditionally in the treatment of STD's.

3.2 Materials and methods

3.2.1 Plant material

Plant material were collected and extracted in different solvents as described in Chapter 2.

3.2.2 Cell lines

MT2 cells were cultured in RPMI 1640 medium (Gibco BRL), containing 10 % fetal bovine serum, 2 mM glutamine, penicillin (50 IU/ml) and streptomycin

(50 µg/ml). MT-2 cells were cultured at 37 ºC in a 5 % CO_2 humidified atmosphere and splinted twice a week. The 5.1 cell line (obtained from Dr. N. Israel, Institut Pasteur, Paris, France) was maintained as MT2 cell line but the media was supplemented with 100 µg/ml G418 (Gibco BRL). Both Hela-Tat-luc and HeLa-Tet-ON cell lines were maintained in DMEM (Gibco BRL) in the presence of 100 µg/ml of hygromycin (Invitrogen) and 100 µg/ml of G418 (Gibco BRL). These cell lines were maintained at 37 ºC in a 5 % CO_2 humidified atmosphere and splinted when confluent.

3.2.3 NF-κB assay

To determine the anti-NF-κB activity of the selected extracts, an NF-κB-dependent luciferase assay was used. The 5.1 cell line was a Jurkat-derived clone stably transfected with a plasmid containing the firefly luciferase gene driven by the HIV-LTR promoter. This promoter is highly dependent on NF-κB activation induced by TNFα. Therefore high expression of luciferase activity reflects NF-κB activation through the canonical pathway.

The assay was done as described by Marquez *et al*., (2005). Briefly, 5.1 cells were pre-incubated with increasing concentrations of the extracts for 30 min and then stimulated with TNFα (2 ng/ml) for 6 h. Then, the cells were lysed in 25 mM Tris phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1 % Triton X-100, and 7 % glycerol. Luciferase activity was measured using an Autolumat LB 953 following the instructions of the luciferase assay kit (Promega) and protein concentration was measured by the Bradford method (Sancho *et al*.,

2004). The background obtained with the lysis buffer was subtracted in each experimental value and the specific transactivation was calculated as RLU/µg protein (relative light units) and the results were expressed as the percent of inhibition where 100 % activity was assigned to transcriptional activity induced by TNFα alone (Campagnuolo *et al.*, 2005). All the experiments were repeated at least four times.

3.2.4 Hela-Tat-Luc assay

To identify potential anti-Tat extracts, another luciferase-based cell system (HeLa-Tat-Luc cells) was used. The HeLa-Tat-Luc cells were stably transfected with the plasmid $pcDNA_3$- TAT together with reporter plasmid LTR-Luc. Therefore the HIV-1 LTR is highly activated in this cell line as a result of high levels of intracellular Tat protein. Cells (10^5 cells/mL) seeded the day before the assay, were treated either with the CDK9 inhibitor DRB, as a positive control, or with the plant extracts at the highest concentration of 50 µg/ml. After 12 h, the cells were washed twice with PBS and the luciferase activity measured as indicated previously for 5.1 cells. All the experiments were repeated at least four times.

3.2.5 Hela-Tet-ON-Luc assay

Compounds considered to be active in both NF-κB (>50 % inhibition) and Tat (>30 % inhibition) assays, were subsequently submitted to evaluation with

a Hela-Tet-ON assay to discard nonspecific luciferase inhibitory activity (Sancho *et al*., 2004).

The cells (10^5 cells/mL) were seeded the day before the assay, and then stimulated with doxycycline (1µg/mL) in the presence or absence of the extracts for 6 h. Then, the cells were washed twice in PBS, lysed and the luciferase activity measured as described (Sancho *et al*., 2004). All the experiments were repeated at least four times.

3.2.6 Cytotoxicity assay

MT2 cells (10^5/ml) were seeded in 96-well plates in complete medium and treated with increasing doses of the extracts for 36 h. Samples were then diluted with 300 µl of PBS and incubated for 1 min at room temperature in the presence of propidium iodide (10 µg/ml). After incubation, cells were immediately analyzed by flow cytometry. All the experiments were repeated at least four times and the results were calculated as a percentage of cell death by GraphPad software. Only extracts of *E. transvaalense* and *Z. davyi* were analysed as they had the best activity in NF-κB and Hela-Tat assays.

3.3 Results and discussion

The extracts were examined for the ability to inhibit NF-κB and Tat proteins which play a role in HIV replication. The toxicity of the active extracts to the cells was also investigated. From the results (Tables 3.1 and 3.2 and Figures 3.1 and 3.2) obtained in this study, the extracts of *Elaeodendron transvaalense, Zanthoxylum davyi* and *Polianthes tuberosa* showed activity in NF-κB and Hela-Tat assays at the highest concentration tested (50 μg/ml). The chloroform extract of *E. transvaalense* showed potent inhibitory activity (> 60 %) at the lowest concentration (1 μg/ml) tested in the NF-κB assay. Both acetone and ethylacetate extracts of *E. transvaalense* also showed a high inhibition, 67 % and 77 % respectively at a 25 μg/ml concentration, whereas *Zanthoxylum davyi* extract demonstrated moderate activity (Figures 3.1 and 3.2).

All the plant extracts were also analysed for their anti-Tat activity in the HeLa-Tat-luc assay. Chloroform and ethyl acetate extract of *E. transvaalense* showed a high Tat inhibitory activity of greater than 70 % at 15 μg/ml. The acetone extract of *E. transvaalense* demonstrated lower activity (>50 %), while *Z. davyi* exhibited moderate activity at 50 μg/ml (Figure 3.2). Those extracts showing anti-NF-κB and anti-Tat activities were also found to be specific in the HeLa-Tet-On assay.

The active extracts were also analysed for cytotoxicity to determine whether the activity was due toxicity. The results (Table 3.3) showed that,

these extracts did not cause significant cell death in the MT2 cell line. The acetone (22.7 %), ethyl acetate (27.6 %) and chloroform (17.1 %) extracts of *E. transvaalense* showed lower cell death percentages after 36 hours at the concentration tested (15 µg/ml). The acetone extract of *Z. davyi* showed little toxicity of 2.4 % cell death. These results indicate that, at the concentrations tested, anti- NF-κB and anti-Tat activity was not due to cellular toxicity.

The use of plant extracts or plant derived synthetic compounds targeting cellular proteins required for efficient HIV-1 replication and transcription has opened new avenues for scientific research in the management of AIDS. According to Marquez *et al*. (2005), plant-derived antiviral compounds interfering with the HIV-1 LTR promoter regulatory proteins are unlikely to generate drug-resistant HIV strains if proven useful for patients.

The results shown here are reported for the first time in these species and suggest that some of the extracts studied might contain chemical compounds that can have an effect on HIV inhibition.

Table 3.1 Results of anti-HIV evaluations for all plant extracts tested at 50 µg/ml

Plant name	Extracts	5.1 NF-κB (Inhi %)[a]	± SD	Hela-TAT-luc (Inhi %)[a]	± SD	Hela-TET-ON-luc (Inhi %)[b]
Anredera cordifolia	70 % acetone	ni		8.70	± 0.00	s
Clerodendrum glabrum	Water	ni		ni		s
Elaeodendron transvaalense	Chloroform	87.80	± 0.17	85.00	± 0.20	s
	Ethyl acetate	50.70	± 0.25	61.10	± 0.13	s
	70 % acetone	34.80	± 0.00	28.70	± 0.24	s
Polianthes tuberosa	70 % acetone	67.40	± 0.30	51.20	± 0.11	s
Rauvolfia caffra	70 % acetone	ni		ni		s
Rotheca myricoides	Water	ni		9.50	± 0.01	s
Senna occidentalis	70 % acetone	5.800	± 0.13	ni		s
Senna occidentalis	Water	ni		ni		s
Senna petersiana	70 % acetone	22.10	± 0.13	ni		s
Terminalia sericea	Chloroform	ni		ni		s
	Ethyl acetate	ni		ni		s
	Water	ni		nt		nt
Zanthoxylum davyi	70 % acetone	50.10	± 0.01	77.00	± 0.2	s

[a] Data are represented as % of inhibition over positive control (i.e., TNF treated cells in 5.1; and untreated in HeLa-Tat- Luc).

[b] S: specific (inhibition < 15%)

nt: not tested.

ni: no inhibition

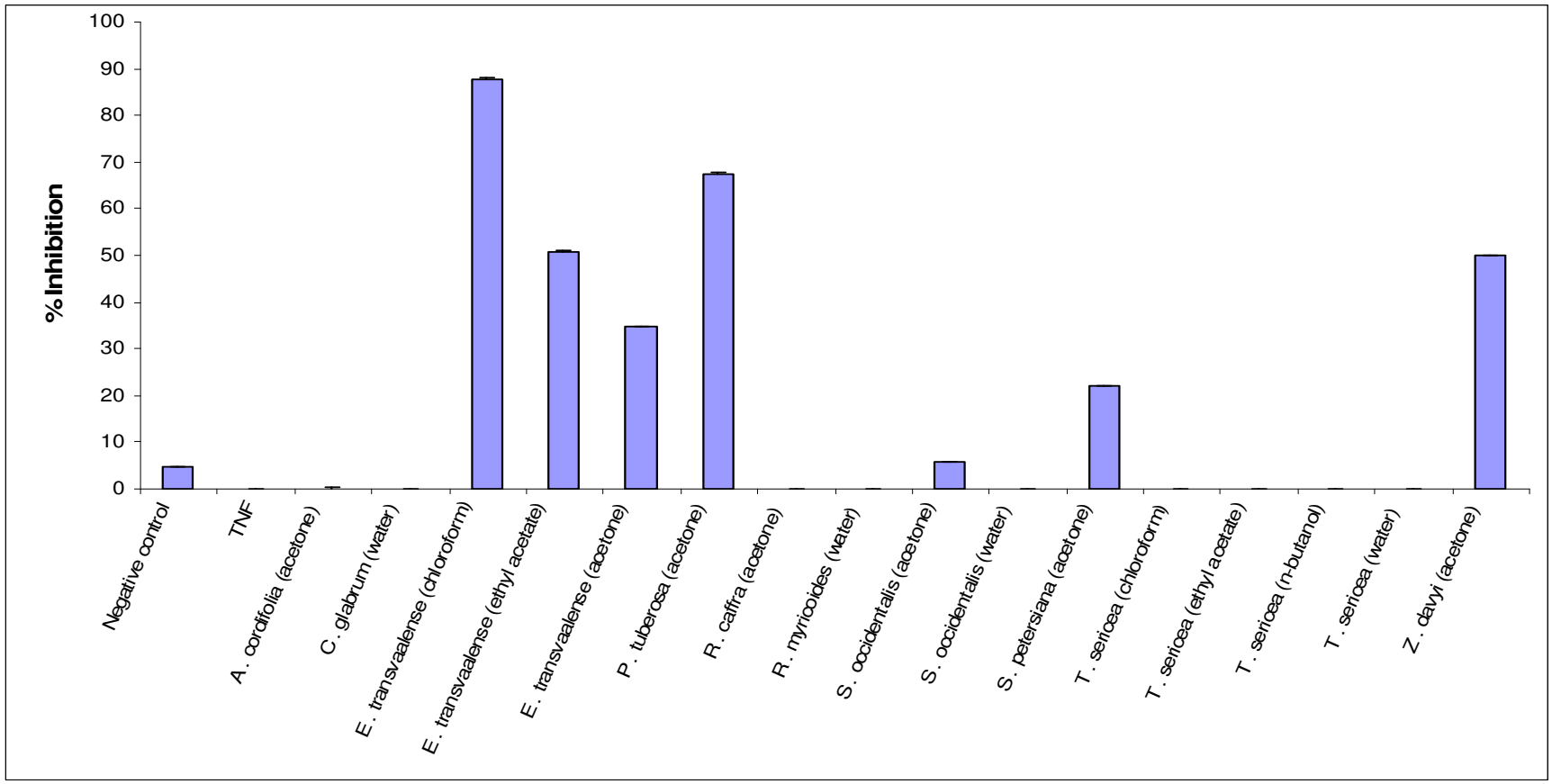

Figure 3.1 Graph showing the anti-NF-κB activity of plant extracts at a 50 µg/ml concentration.

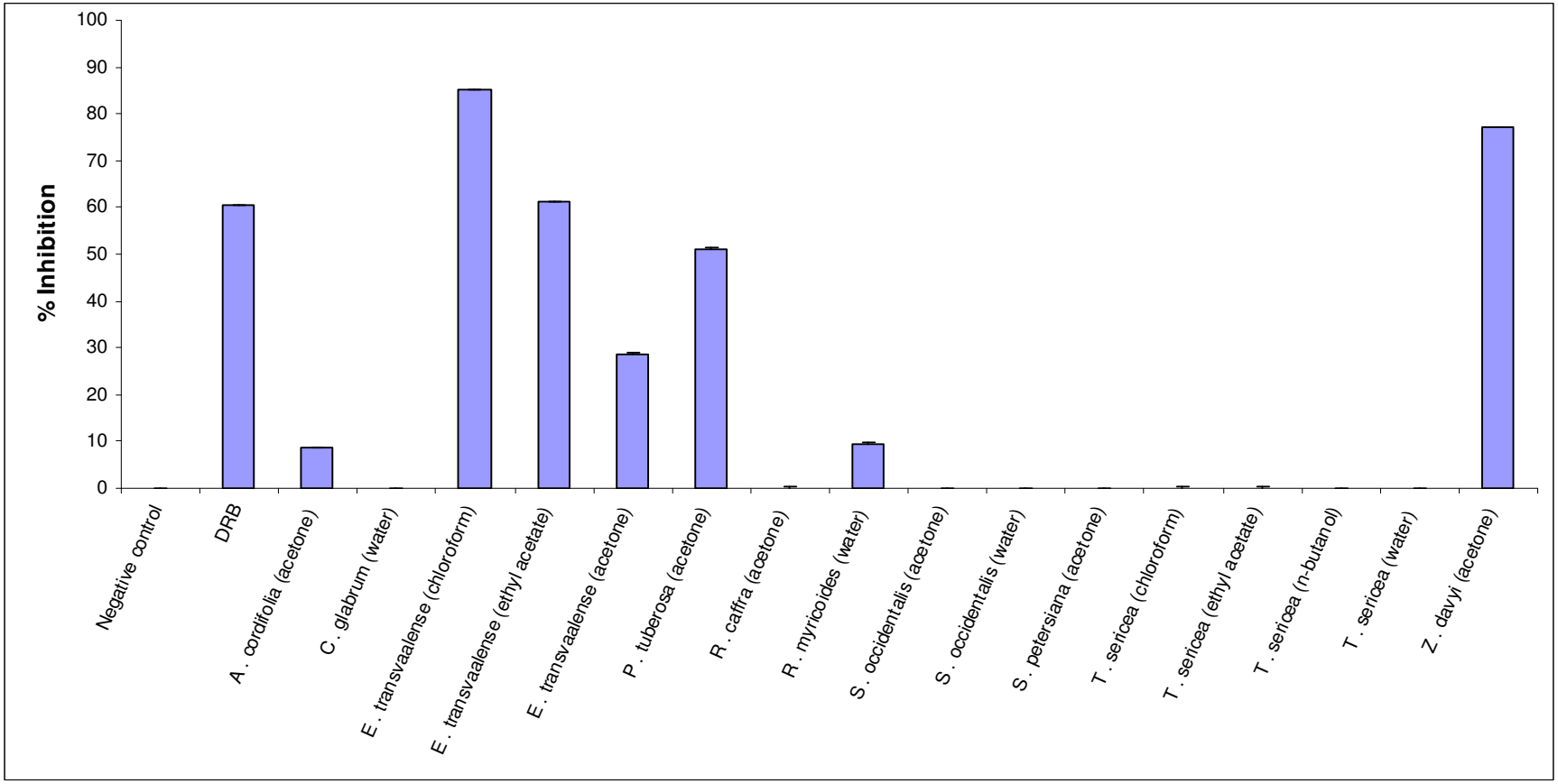

Figure 3.2 **Graph showing the anti-Tat activity of plant extracts at a 50 µg/ml concentration.**

Table 3.2 Results of anti-HIV evaluations of plant extracts that showed activity

Plant	Extracts	5.1 NF-κB (Inhi %)[a]				HeLa- Tat-Luc (Inhi %)[a]			HeLa- Tet-ON (Inhi %)[a]
		Concentration (µg/ml)							
		1	5	15	25	1	5	15	50
E. transvaalense	70% Acetone	0.0 ± 0.2	45.0 ± 0.3	54.0 ± 0.2	67.0 ± 0.4	0.0 ± 0.0	22.0 ± 0.0	43.0 ± 0.1	S
E. transvaalense	Chloroform	64.0 ± 0.6	64.0 ± 0.4	64.0 ± 0.1	73.0 ± 0.2	28.0 ± 0.1	66.0 ± 0.0	76.0 ± 0.2	S
E. transvaalense	Ethyl acetate	79.0 ± 0.3	72.0 ± 0.4	75.0 ± 0.0	77.0 ± 0.2	63.0 ± 0.0	66.0 ± 0.2	75.0 ± 0.0	S
Z. davyi	70% Acetone	34.0 ± 0.1	48.0 ± 0.3	54.0 ± 0.1	57.0 ± 0.1	1.4 ± 0.1	25.0 ± 0.0	50.0 ± 0.2	S

[a] Data are represented as % of inhibition over positive control (i.e., TNF treated cells in 5.1; and untreated in HeLa-Tat- Luc).

[b] S: specific (inhibition < 15%).

Table 3.3 Cell death (necrosis) percentage at 6, 24 and 32 hours intervals.

Plant	Extracts	Concentration (µg/ml)	6 hrs	24 hrs	32 hrs
Control			1.5 ± 0.0	2.1 ± 0.0	2.2 ± 0.5
E. transvaalense	acetone	1.0	2.1 ± 0.0	2.4 ± 0.5	2.7 ± 0.3
		5.0	2.7 ± 0.1	7.6 ± 0.2	10.9 ± 0.0
		15.0	4.2 ± 0.7	18.1 ± 0.0	22.7 ± 0.2
	ethyl acetate	1.0	2.0 ± 0.1	2.8 ± 0.1	4.8 ± 0.2
		5.0	3.4 ± 0.2	8.6 ± 0.6	14.5 ± 0.2
		15.0	2.6 ± 0.1	13.2 ± 0.2	27.6 ± 0.1
	chloroform	1.0	2.2 ± 0.0	9.5 ± 0.0	3.9 ± 0.5
		5.0	2.7 ± 0.3	7.6 ± 0.1	12.1 ± 0.3
		15.0	5.5 ± 0.1	14.7 ± 0.1	17.1 ± 0.0
Z. davyi	acetone	1.0	1.5 ± 0.4	1.9 ± 0.3	2.1 ± 0.2
		5.0	1.4 ± 0.2	1.6 ± 0.4	1.9 ± 0.1
		15.0	1.4 ± 0.2	1.8 ± 0.2	2.4 ± 0.1

3.4 References

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